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Research Note

Effects of Reduced Oxygen Atmosphere on Motility, Penetration of Host Cells, and Intracellular Survival of *Eimeria nieschulzi* Sporozoites in Vitro

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ABSTRACT: We compared the ability of sporozoites of the rat coccidian, *Eimeria nieschulzi,* to become motile, penetrate Madin-Darby bovine kidney cells, and remain viable within host cells for up to 15 hr both in candle jars and in a 5% CO_2/95% air incubator. Results showed both motility and invasion of host cells to be unaffected by atmosphere whereas longer term survival was enhanced in the presence of a reduced oxygen atmosphere.

KEY WORDS: *Eimeria nieschulzi,* Apicomplexa, coccidia, blind well chamber, in vitro, oxygen.

Tilley and Upton (1988) reported development of the rat coccidian, *Eimeria nieschulzi* Dieben, 1924, to be enhanced when cultures were grown under reduced oxygen concentrations. Fukata et al. (1992) provided one explanation for this phenomenon by showing that reducing the oxygen concentration results in greater numbers of intracellular sporozoites of the chicken coccidian, *E. tenella.* Both Ricketts (1992) and Wrede et al. (1993) have found reduced O_2 to have no effect on growth of *E. tenella* in vitro, and Wrede et al. (1993) concluded that O_2 concentration affects only host cell invasion and not asexual development. Our recent studies, however, have indicated that this is not so for *E. nieschulzi,* the results of which are presented here.

Oocysts of *E. nieschulzi* were obtained from experimentally infected rats, strained through a graded series of sieves, sporulated, and stored in an aqueous 2.5% (w/v) $K_2Cr_2O_7$ solution as described by Bristol et al. (1983). Prior to use, oocysts were concentrated by sucrose flotation (Barnard and Upton, 1994) and incubated for 15 hr at 4°C in 100% Clorox® bleach to weaken oocyst walls (Hosek et al., 1988). Oocysts were then washed 3× in distilled water and 2× in phosphate-buffered saline (PBS) by centrifugation. Sporocysts were liberated from oocyst walls by grinding oocysts in a hand-held, Ten-Broeck glass-ground tissue grinder (Fisher Scientific, Pittsburgh, Pennsylvania). Free sporozoites were obtained by incubating sporocysts for 45 min in an excystation solution consisting of 0.25% (w/v) trypsin–0.75% (w/v) sodium taurocholate in PBS at 37°C. For migration assays (later), sufficient units of soybean trypsin inhibitor (Sigma Inc., St. Louis) dissolved in PBS were added to the mixture to neutralize 100% of the trypsin. Sporozoites were then purified from debris by nylon wool column filtration (Tilahun and Stockdale, 1982), washed by centrifugation at 800 *g* 1× in PBS and 2× in either Dulbecco's modified Eagle's medium (DMEM) for migration assays or RPMI 1640 for cell penetration assays. Mean numbers of sporozoites were calculated in each experiment by hemacytometer. All parasites were <2 mo old when utilized.

To assess whether or not motility of sporozoites was affected by atmosphere, sporozoite migration assays were performed as described by Upton and Tilley (1992). Briefly, after sporozoites were purified by filtration through a nylon wool column, 200 μl of DMEM containing 5.0 × 10^5 sporozoites was added to the top chamber of each blind well chamber (NeuroProbe Inc., Cabin John, Maryland). Sporozoites were separated from chemoattractant in the bottom chambers by a cellulose/acetate millipore filter with a pore size of 8.0 μm (Millipore Inc., Bedford, Massachusetts). Chambers were then divided into 2 equal groups and incubated at 37°C in either a 5% CO_2/95% air incubator or in candle jars (Tilley and Upton, 1988; Upton et al., 1991, 1994; Upton and Tilley, 1992). The O_2 concentration in candle jars and the 5% CO_2/95% air incubator was measured at 16.3–16.9 and 19.6%, respectively (Upton et al., 1994). Candle jars were preheated to 37°C prior to use to more carefully mimic the situation within the 5% CO_2/95% air

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incubator where cultures rise to incubator temperature rapidly. Positive chemoattractant consisted of 10% fetal bovine serum (FBS) in DMEM, and negative controls employed 100% DMEM only. The number of parasites that migrated through the filters and collected in the bottom chambers after 3 hr was assessed by counting sporozoites by hemacytometer (Upton and Tilley, 1992). Each test solution was examined at least in triplicate and the contents from each test well counted 10× (i.e., 10 counts/well × ≥3 wells = ≥30 counts per test solution).

Although long-term development of *E. nieschulzi* in Madin-Darby bovine kidney (MDBK) cells has been shown to be enhanced by reduced oxygen concentrations (Tilley and Upton, 1988), the effects of reduced O_2 on parasite survival in culture has been unknown. Therefore, we assessed parasite survival following penetration of host cells at 3 and 15 hr postinoculation using 2 different O_2 concentrations. MDBK cells (MDBK [NBL-1]; ATCC CCL 22) were grown to confluency on 22-mm² coverslips (VWR Scientific, San Francisco) in 6 well cluster plates (Costar Inc., Cambridge, Massachusetts). Media consisted of RPMI 1640 supplemented with 10% FBS, 10 mM HEPES, Na-bicarbonate to pH 7.4, 100 IU penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone. Prior to inoculation into cultures, each well was washed 2× with PBS and then inoculated either with 1.0×10^6 sporozoites for 3-hr incubations or 1.0×10^5 sporozoites for 15-hr incubations. These intervals were chosen because by 3 hr large numbers of sporozoites had penetrated cells but nonviable parasites had not yet degenerated; at 15 hr, sporozoites had not yet differentiated into first generation meronts, but nonviable sporozoites in cells were degenerating and could be distinguished from viable intracellular forms. Prior to inoculation, sporozoites were suspended evenly in the cell culture medium for uniform distribution over cells. Plates were incubated either in candle jars or in a 5% CO_2/95% air incubator. Coverslips were removed from wells using forceps, washed vigorously in a beaker of PBS to remove extracellular sporozoites, and examined by Nomarski interference contrast microscopy. The number of intact, intracellular sporozoites was then counted in 25, × 40 fields for each coverslip. Each experiment was replicated 4–8×. Total number of intracellular sporozoites in cells within each entire well was then calculated based on the assumption that monolayers were confluent. Results of all experiments were compared using the Wilcoxen Mann-Whitney *U*-test. Results are expressed as means followed by ± standard deviations.

Table 1. Effect of atmosphere on migration of sporozoites of *Eimeria nieschulzi* through a porous filter.

Attractant*	Replicates (*N*)	Number of migrating sporozoites ($\bar{x}$ ± SD)	% migration
5% CO_2/95% air			
100% DMEM	6	67 (163)	0.013
10% FBS in DMEM	6	123,733 (9,981)	24.7
Candle jars			
100% DMEM	6	400 (438)	0.08
10% FBS in DMEM	5	115,760 (12,217)	23.2

* $P > 0.05$ between same groups.

A reduced oxygen atmosphere in candle jars did not significantly affect motility of sporozoites in the blind well chambers (Table 1) ($P > 0.05$). Nearly 25% of the sporozoites migrated through the filters in the reduced oxygen atmosphere, whereas over 23% of the sporozoites did so in the 5% CO_2/95% air atmosphere. Likewise, atmosphere had no effect on penetration of sporozoites into host cells (Table 2) ($P > 0.05$). Over 22% of the sporozoites penetrated cells in both atmospheres. In contrast to the short-term studies, however, survival of sporozoites was affected by atmosphere after 15 hr (Table 2). Less than 39% of the sporozoites appeared intracellular and viable (nondegenerate) in the 5% CO_2/95% air atmosphere; many others were intracellular but appeared in various phases of degeneration. Approximately twice as many were observed to be viable in cells incubated in candle jars. This difference was significant ($P < 0.05$).

These results demonstrate that the effects of low O_2 on sporozoites of *E. nieschulzi* are different than for *E. tenella*. Sporozoite motility and host cell penetration of the former do not appear to be atmosphere-dependent, at least under the conditions of this study. However, survival within host cells, at least in the short term, are affected by atmospheric O_2. These differences may be due to a variety of factors, including differences in the class of vertebrate infected, site of invasion, and types of host cells targeted. The ability of many *E. nieschulzi* sporozoites to actively invade cells only to die later may be explained in

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Table 2. Effect of atmosphere on penetration and survival of sporozoites of *Eimeria nieschulzi* in MDBK cells in vitro.*

3 hr postinoculation		15 hr postinoculation	
Candle jars ($N = 6$)	5% CO_2/95% air ($N = 8$)	Candle jars ($N = 6$)	5% CO_2/95% air ($N = 8$)
222,608 (40,128)	223,011 (39,069)	76,662† (4,726)	38,560† (2,260)

* 1.0×10^6 (3-hr study) or 1.0×10^5 (15-hr study) sporozoites inoculated/well onto monolayers of MDBK cells on 22-mm^2 coverslips. Coverslips were examined 3 or 15 hr postinoculation and the mean number of sporozoites/25 ×40 objective fields/coverslip counted. Results are expressed as the projected mean number of sporozoites/well (±SD).

† $P < 0.05$ between groups.

several ways. For instance, prolonged exposure of *E. nieschulzi* sporozoites to high oxygen concentrations may result in eventual, rather than immediate, sporozoite death. Eimerian sporozoites are known to have low levels of oxygen scavenging enzymes (Hughes et al., 1989; Michalski and Prowse, 1991), which may be exhausted relatively quickly and in an oxygen-dependent manner during the penetration process. Alternatively, lower oxygen concentrations may enhance the ability of sporozoites to successfully establish a functional parasitophorous vacuole by affecting extrusion of rhoptry contents. Tachyzoites of *Toxoplasma gondii* are known to be capable of invading host cells with or without extruding rhoptry contents, but these latter organisms are thought to eventually die and degenerate (Silva et al., 1982). Finally, Upton et al. (1994) recently showed that host cell type plays an important role in survival of *Cryptosporidium parvum* under different atmospheres in vitro. In these studies, survival of *C. parvum* in MDBK cell was enhanced in a reduced O_2 atmosphere, whereas higher numbers of parasites were found in a 5% CO_2/95% atmosphere when human HCT-8 cells were employed. These results suggest that the observed effects may be due to lower O_2 on host cells rather than parasites, which would in turn affect survival of intracellular parasites.

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